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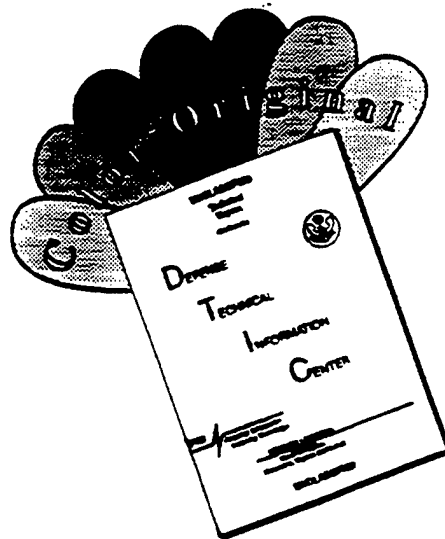
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Breast epithelial cell function is greatly influenced by interactions with the underlying basal lamina. Matrilysin, a matrix metalloproteinase, has previously been shown to be expressed in both adenomas and carcinomas of the human breast. We have tested the hypothesis that cell-ECM interactions regulate the expression of matrilysin in human breast carcinoma cells *in vitro*. We have used northern analysis and *in situ* hybridization to determine levels and localization of several members of the MMP family in human tumors implanted into nude mouse mammary glands. Tumors from mammary glands injected with the human breast adenocarcinoma cell line MDA-MB-468 were shown to express matrilysin; a MMP that is primarily expressed in normal and neoplastic cells of epithelial origin. Stromelysin-1 was induced in the stroma of the host mammary gland in the region immediately surrounding the tumor. Northern analysis revealed that gelatinase A, which was not produced by MDA-MB-468 *in vitro*, was expressed in the tumor. This combination of MMPs along with stromelysin-3, which has been extensively studied in breast cancer, may lead to the eventual metastasis of mammary tumors to the regional lymph nodes and distant sites. We also describe a new antibody against human matrilysin which indicates that matrilysin is expressed in epithelial cells as expected and appears to be apically secreted in endometrium, breast and prostate. This lumenal secretion of an MMP that degrades extracellular matrix molecules may have important implications as to its function in normal and neoplastic tissues.

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FOREWORD

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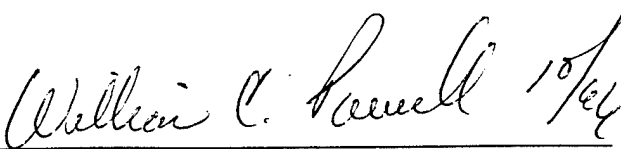
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Introduction:

Breast cancer is the most commonly diagnosed female cancer in the United States population with an estimated 182,000 new cases in 1993 (32% of all female cancers diagnosed in 1993) (1). Metastatic spread to the regional lymph nodes has long been used as a predictor of the extent of disease and with reasonable success. When the tumor has invaded the regional lymph nodes the 5-year survival rate drops from 93% to 73% and when distant metastasis is found the rate falls to 19% (1). These data make it clear that the process of tumor cell invasion and metastasis are important in the progression of breast cancer from a benign to a malignant state. The process by which neoplasms metastasize is composed of a complex series of events (2, 3). One of the initial steps in tumor cell invasion is the degradation of the basal lamina (BL) and local invasion of the surrounding tissue. To move through the BL, cells must secrete proteinases that are able to degrade the components of the BL including, collagen type IV, laminin, tenascin and entactin.

The matrix metalloproteinases (MMPs) are a multigene family of secreted proenzymes whose substrates are the proteins that make up the BL and the extracellular matrix (4). Tight regulation of the expression and activity of MMPs and their specific inhibitors, TIMPs, maintain cells in a non-invasive phenotype (2). A change altering the balance in favor of MMP activity may be one of the initial steps in tumor cell invasion. Matrilysin is the smallest known member of the MMP family containing only the signal sequence, the pro-peptide and the active site. Matrilysin has been shown to be expressed in several different human tumor types including: breast, prostate, colon, gastric, rectal and head and neck (for review see(5)). Matrilysin degrades casein, gelatins I, III, IV and V, fibronectin, laminin, elastin and entactin. Matrilysin also interacts with other proteinases; specifically, matrilysin can cleave activated interstitial collagenase to increase collagenases activity five fold (6). Matrilysin can proteolytically cleave pro-urokinase to yield low-molecular weight pro-urokinase that is unable to bind to its specific transmembrane membrane receptor (7, 8). Matrilysin mRNA has been shown to be expressed in a high percentage of both adenomas and carcinomas of the breast (9, 10). *in situ* analysis indicates that the mRNA is localized to the transformed epithelial cells of the breast (10). I have recently shown that matrilysin expression in prostate tumor cells increases the invasive potential of those cells (11).

Mammary epithelial cells have been shown to be highly dependent upon the basal lamina to which the basal aspect of the epithelial cells are attached (12). It has become apparent that changes in the BL or alterations in the way breast epithelial cells respond to normal BL may change the protein expression patterns of the epithelial cells. There is a growing body of literature that indicates that the integrin family of ECM receptors is capable of transducing a signal derived from the ECM to the nucleus and thus alter mRNA and protein production. One of the first instances of signal transduction through an integrin receptor was described by Werb et.al. They showed that proteolytic fragments of fibronectin and Arg-Gly-Asp containing synthetic peptide fragments can transduce a signal through the fibronectin receptor and increase the expression of Interstitial procollagenase and prostromelysin-1 in rabbit synovial fibroblasts (13). It has also been shown that a synthetic peptide derived from the laminin A chain can increase the amount of collagenase IV activity (14). Recently it has

been shown that both cell adhesion and integrin clustering causes the phosphorylation of a tyrosine kinase (pp125^{FAK}) associated with focal adhesion contacts (15, 16).

It is of interest to understand the regulation of matrilysin in breast cancer where invasion and metastasis has such a large effect on prognosis. We hypothesize that alterations in the interactions between breast epithelial cells and the BL can modify the invasive and/or metastatic phenotype of the transformed breast epithelial cells. Since the integrin family of receptors are the primary mode of cellular attachment to the BL and they have been shown to transduce signals that effect gene regulation, integrin mediated signal transduction is the most likely method to regulate MMP expression.

In the previous annual report we described data indicating that matrilysin mRNA production by MDA-468 cells could be inhibited by the presence of collagen type I and that plating on other matrix molecules, such as fibronectin, laminin and vitronectin, had no apparent effect. MCF-7 cells plated on these same matrices failed to induce the matrilysin mRNA. We also reported that intramammary injection of MDA-468 cells into nude mice developed into tumors which induced stromelysin-1 mRNA in the host stroma. The tumor cells produced matrilysin and MT1-MMP, a new member of the metalloproteinase family. In this progress report extensions of these studies will be discussed.

Body:

During the past year the focus of this project has followed 2 lines of investigation, characterization of the interactions between tumor cells and host stroma and the development and characterization of an anti human matrilysin antibody that is useful for western analysis and immunohistochemistry.

Due to the relative lack of useful antibodies against mouse MMPs we have used in situ hybridization to localize the production of MMPs in the nude mouse xenograft system. The xenograft approach has allowed us to answer several questions regarding the effects of host tumor interactions on the production of MMP in the interface between tumor and host tissues. we have expanded the initial study described in the previous report to include not only MDA-468, but also MCF-7 and MDA-231. The use of these 3 cell lines was designed to answer the question do breast cancer cells at different stages of progression utilize the same sets of MMPs produced by the host stroma. The results of this study are summarized in Table I.

Table I

MMP	MCF-7	MDA-468	MDA-231
Matrilysin	None	Tumor	None
Stromelysin-1	Stroma near tumor	Stroma near tumor	Stroma near tumor
Stromelysin-2	ND	Infil. Immune cells	ND
Stromelysin-3	None	Tumor stroma	Stroma near Glands
Collagenase	Tumor stroma	Stroma near tumor	None
Gelatinase A	Tumor stroma	Tumor stroma	Tumor stroma
TIMP-1	ND	Stroma near invasion	ND
TIMP-2	ND	Stroma near tumor	ND
TIMP-3	ND	Stroma near tumor	ND

The expression of stromelysin-1 in this model was somewhat surprising. Strong expression near the tumor is a classic pattern for stromelysin-3 in human breast cancer, but this has not been reported before for stromelysin-1. This expression of stromelysin-1 could be induced through tumor production of soluble growth factors and cytokines that affect fibroblast MMP production. MDA-MB-468 cells are known to overexpress the EGF receptor as well as secrete TGFa which could form an autocrine loop and result in the production of matrilysin by MDA-MB-468 cells. Data from our lab in collaboration with Dr. Carlos Arteaga indicates that matrilysin expression in MDA-MB-468 cells may require the EGF receptor. This was shown by examining matrilysin expression in MDA-468 cells that have had their EGFR levels reduced by either antisense RNA or by treatment with a TGFa/endotoxin fusion protein. To determine if the expression of stromelysin-1 in the host stroma was due to a soluble factor secreted by MDA-468 cells we treated human foreskin fibroblasts with serum free conditioned media from MDA-468 cells. Northern analysis was performed on total RNA from the HFF cells (Figure 1)
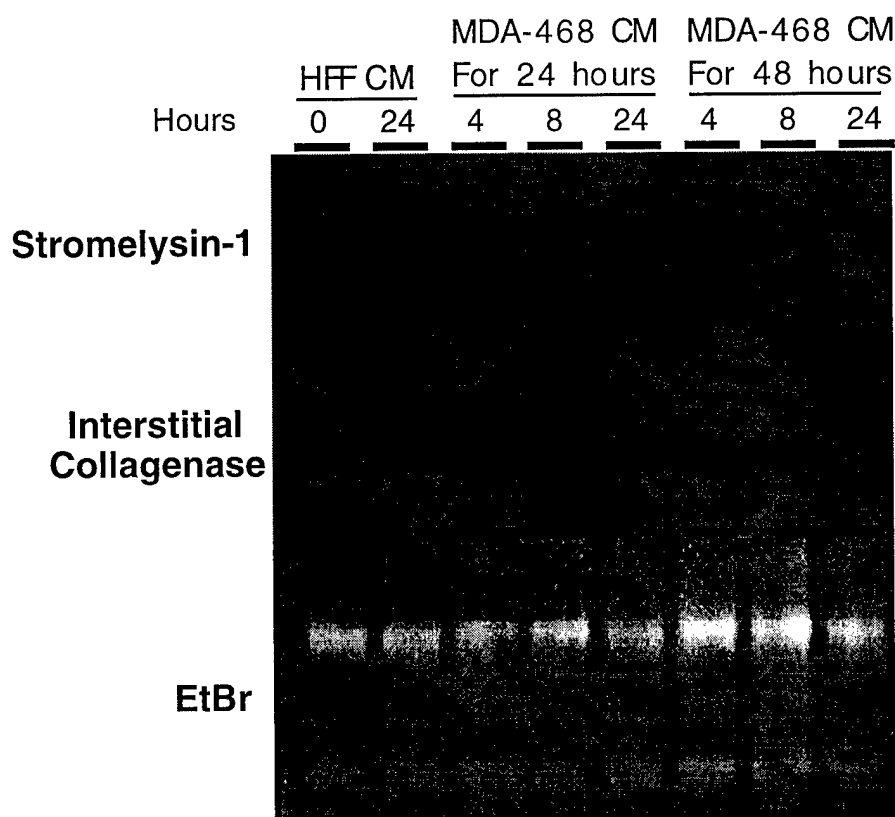

Figure 1. Northern analysis of human foreskin fibroblasts treated with MDA-MB-468 cell conditioned media for stromelysin-1 and interstitial collagenase. The cells were treated for the indicated times and total RNA was isolated. Stromelysin-1 and interstitial collagenase are both up regulated in a dose dependent manner in response to a soluble factor secreted by MDA-MB-468 cells.

Induced expression of stromelysin-1 and collagenase by serum free conditioned media indicates that there may be a soluble factor that is secreted

by MDA-468 cells that can upregulate the transcription of these two genes. TGFa has been shown to induce the expression of MMPs in various model systems (17, 18). Subsequent experiments to determine if this is due to either EGF or TGFα have failed to implicate either of these two molecules. We have used neutralizing antibodies to both EGFR and to TGFa to block this effect and have not seen a significant decrease in the level of either stromelysin-1 or collagenase (data not shown). Future experiments will attempt to elucidate the factor responsible for in vitro stimulation of stromelysin-1 in fibroblasts.

The second main direction of this research was driven by the need for a better antibody against human matrilysin for use in western analysis as well as for immunohistochemistry. In collaboration with Dr. John Couchman we have produced a rat monoclonal antibody against the full length human matrilysin protein. The rat was chosen as the host for the monoclonal generation so that the antibody could be used on both human an mouse tissue. The initial characterization was performed by immunohistochemistry on sections of MDA-468 tumors in nude mice since it was known that human matrilysin was expressed in vivo (Figure 2)
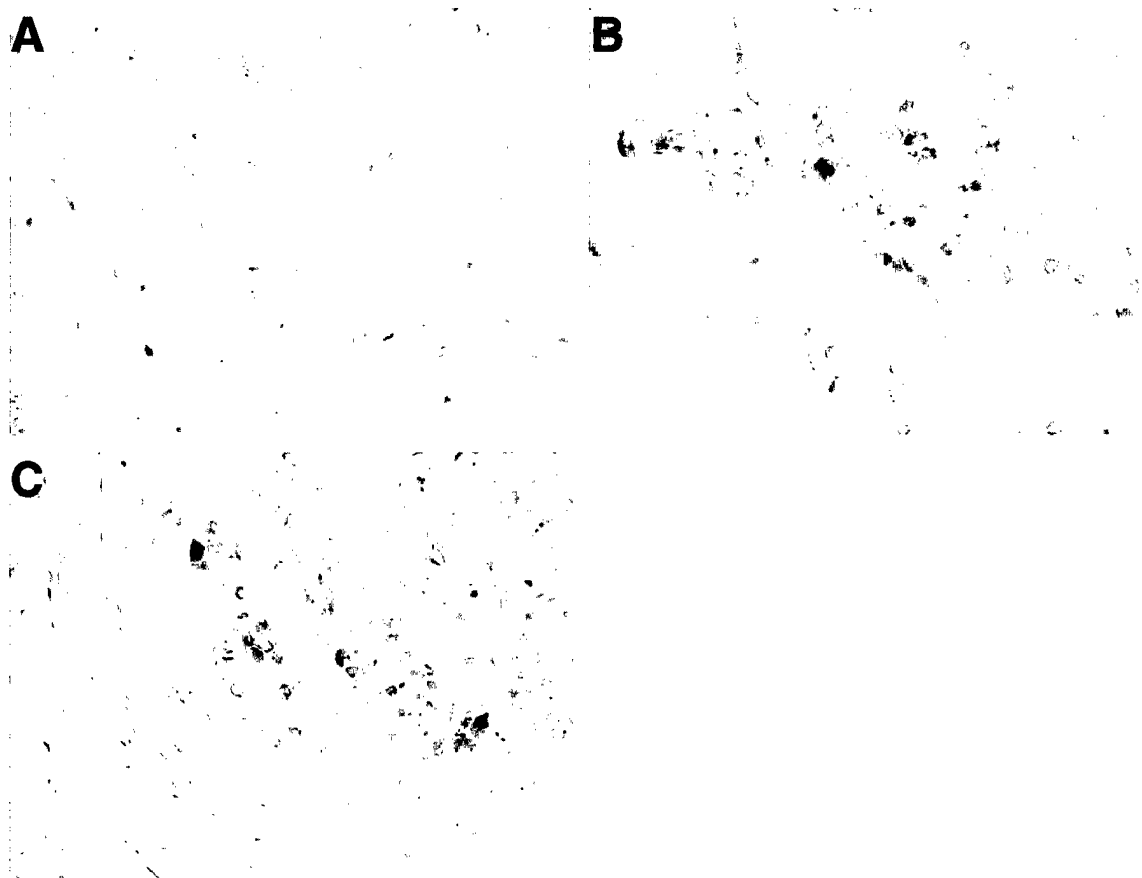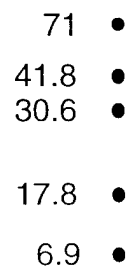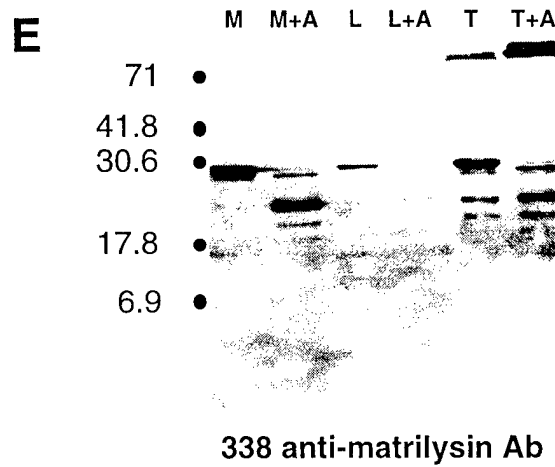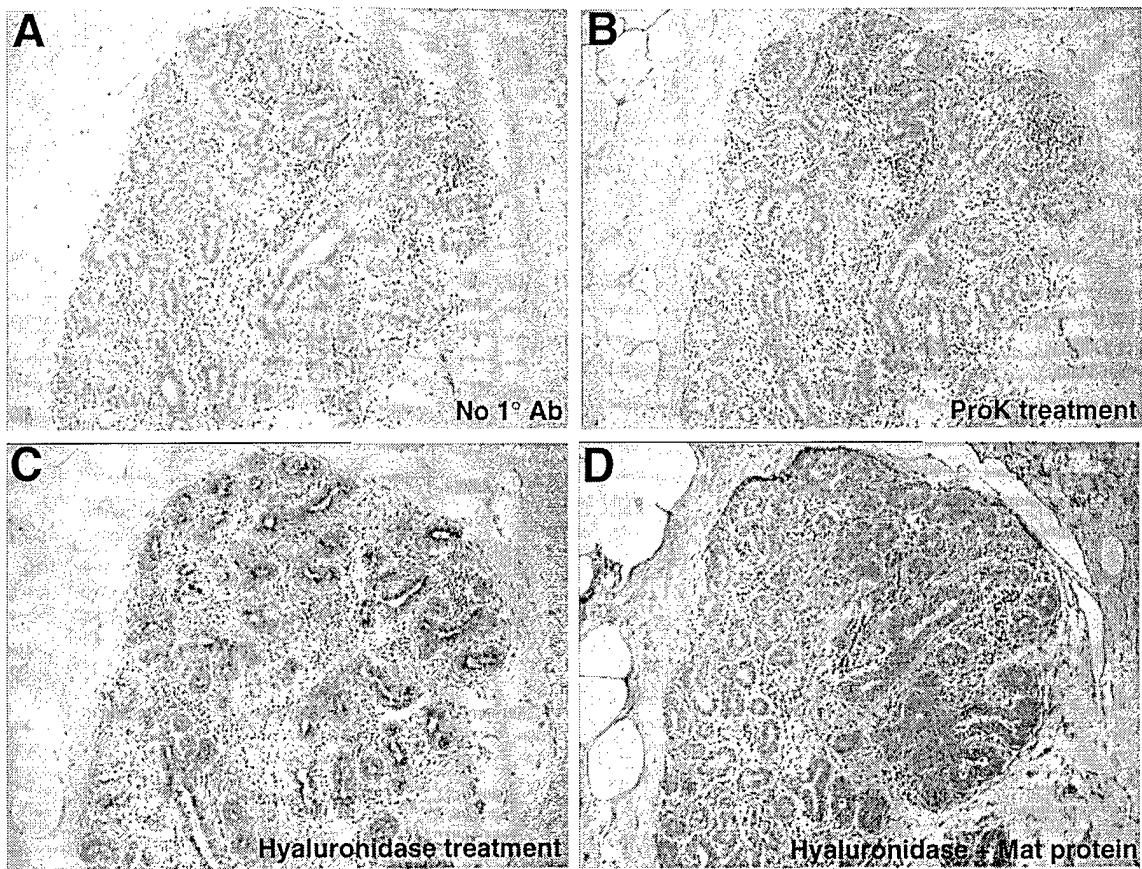

Figure 2. Characterization of rat monoclonal antibodies. Panel A shows the typical negative staining of 25 of the 26 monoclonals tested. Panel B shows the staining of monoclonal supernatant #338 using the TrueBlue peroxidase substrate (blue staining with

red nuclear counter stain). Panel C shows human matrilysin staining using the ascites generated from the #338 hybridoma.

This antibody is by far the best generated against human matrilysin that our laboratory has seen. There is one unusual characteristic about either the antibody or the epitope that I recognizes and that is that the protocol requires treatment with hyaluronidase for recognition of the epitope (Figure 3C). Other antigen retrieval techniques including microwave and proteinase K treatment failed to produce positive staining (Figure 3B). The staining was shown to be specific by pre binding purified human matrilysin to the ascites fluid prior to addition to the tissue (Figure 3D). This antibody also works well on western analysis of conditioned media, tissue culture cell lysates and proteins extracted form tumors (Figure 3E).

Figure 3. (Next Page) Further characterization of the matrilysin antibody. Immunohistochemistry was performed on tissue from human reduction mammoplasties with the following treatments: A - no primary antibody added. B - tissue treated with proteinase K prior to addition of the 338 antibody. C - Hyaluronidase treatment instead of proteinase K. D- Antibody preabsorbed to antigen prior to addition to tissue. Panel E shows western analysis of MDA-468 conditioned media (M), MDA-468 cell lysates (L) or protein extracted from a MDA-468 nude mouse tumor (T). Each one of these samples was run as is or treated with APMA which causes autoactivation and a decrease in size to a 20 kDa form.

A — No 1° Ab

B — ProK treatment

C — Hyalurohidase treatment

D — Hyaluronidase + Mat protein

E

	M	M+A	L	L+A	T	T+A
71						
41.8						
30.6						
17.8						
6.9						

338 anti-matrilysin Ab

71
41.8
30.6

17.8

6.9

No 1° antibody

We then wanted to determine if the antibody would recognize human matrilysin in tissues from our Pathology Dept. In tissues where we have already performed matrilysin in situ hybridizations (Figure 4). The protein and mRNA do indeed co-localize in a very specific manner indicating that the antihuman matrilysin antibody generated is specific. Interestingly, localization of matrilysin was in the epithelial cells as expected, but it appeared to secreted into the lumenal compartment rather than basally as would be expected for an enzyme that degrades extracellular matrix molecules. This was a very surprising result and warranted further investigation. It could have been that the tissue tested, reduction mammoplasties, was not truly normal and matrilysin secretion could have been altered.
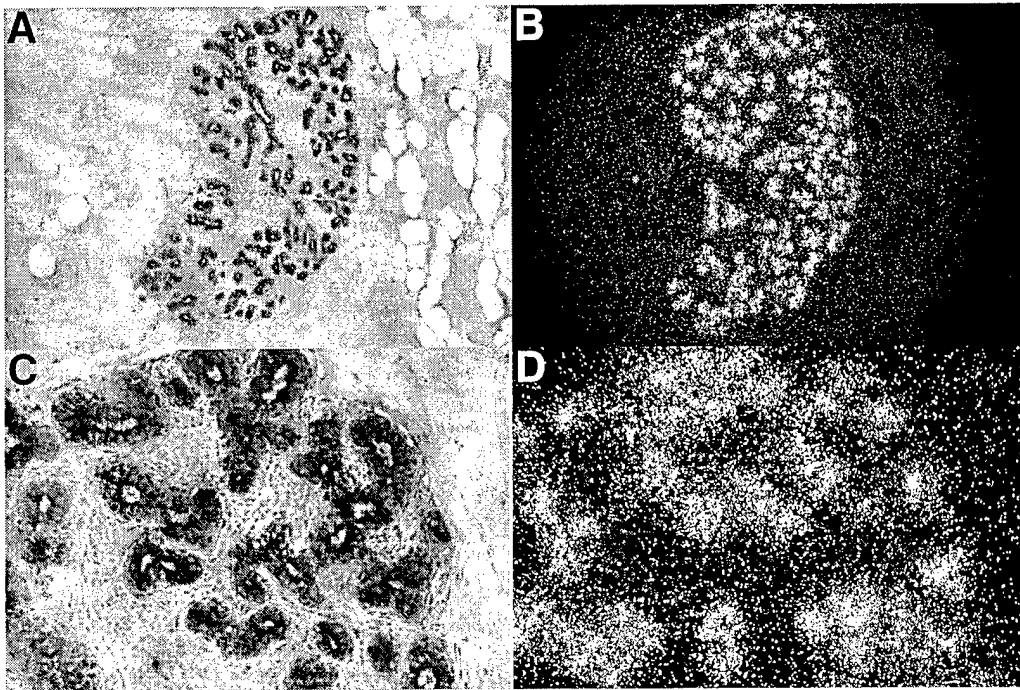

Figure 4. Co-localization of matrilysin mRNA and protein in human tissue. Panels A and C show immunohistochemical localization of the human matrilysin protein in reduction mamoplasty tissue. Panels B and D show in situ localization of the matrilysin mRNA in a serial section. Panels C and D are higher power magnifications of A and B.

In collaboration with Dr. Kevin Osteen in our Dept. We localized matrilysin in normal human endometrial tissue which has been shown previously to express matrilysin (Figure 5 A-D). The late proliferative phase tissue was very informative, the high power magnification shows that matrilysin can be secreted both basally and apically into the lumen. The secretion apparently switches some time before menstruation because it is seen only apically in menstrual tissue. All other normal tissues examined so far human and mouse exhibit apical secretion (prostate, seminal vesicles, Paneth cells). Through a collaboration with Dr. David Page at our institution we have begun to

look at a wide variety of human breast cancer cases (figure 5 E-G). These panels are a representation of what we have seen after having looked at greater than 50 cases. If matrilysin is found in the tissue, it is most often found in normal ducts and glandular units. The most consistent finding is that matrilysin is seen in the normal ducts and glands adjacent to expanding lesions such as hyperplasias and papilomas where the surrounding tissue is significantly deformed by the neoplasia. We do see expression in some invasive tumors and that expression visually looks very similar to the expression in MDA-468 tumors in nude mice.

Figure 5. (Next Page) Matrilysin immunohistochemistry on human endometrial tissue and breast cancers. Panels A and B are human endometrium in the late proliferative phase. Panels B and C are human menstrual tissue. Panels B and D are higher magnifications of A and C. Panel E is a normal duct found near a micro papiloma. Panel F shows matrilysin staining in a normal glandular unit adjacent to an expanding hyperplasia (P). Panel G shows matrilysin expression in a metaplastic spindle cell carcinoma. Human breast cases were obtained through a collaboration with Dr. David Page in the Pathology Dept. at our institution.
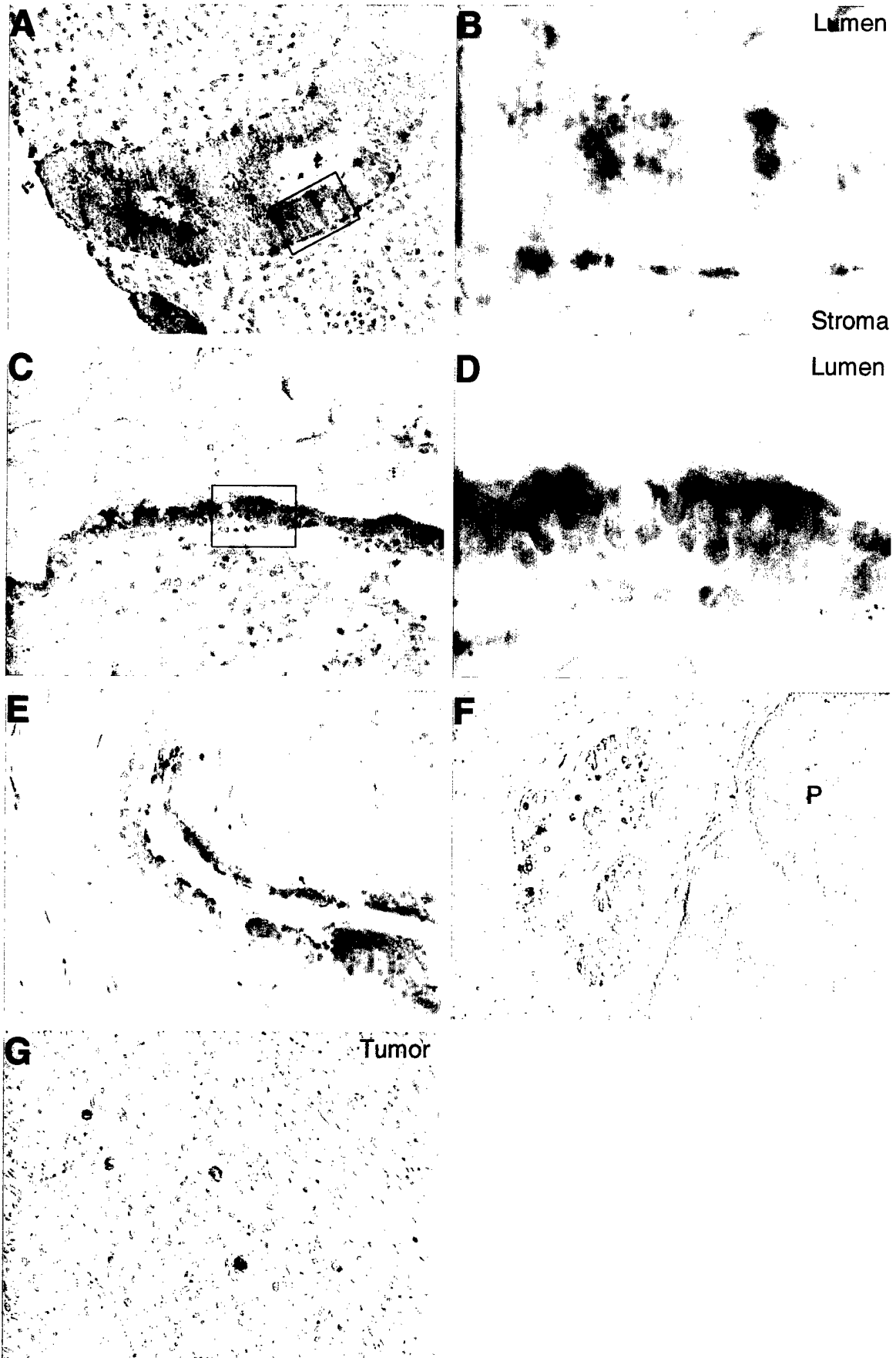
Conclusions:

Through the use of the xenograft system we have been able to ascertain a number of characteristics of the host/tumor interface with respect to MMP expression. One of the questions raised in the first report was whether a epithelial to mesechymal transition (19) occurs in these tumors i.e. do tumor cells become more like fibroblasts and begin producing stromal MMPs such as stromelysin-1, 3, gelatinase A and collagenase. Our data using human and mouse specific in situ probes shows that this transition does not occur in this model system. The interaction appears to be due to the presence of the tumor or a response to a factor that the tumor cells are producing. The ability· of conditioned media from MDA-MB-468 to induce stromelysin-1 and collagenase in human fibroblasts points to a soluble factor secreted by MDA-MB-468 cells, that may be responsible for the in vivo induction of stromelysin-1 in the mouse mammary gland. We have shown that there are several MMPs as well as TIMPs produced in this model system that function to degrade ECM and BM proteins to allow for invasion with subsequent metastasis. Understanding interactions between tumor cells and normal surrounding tissue may aid in the evaluation of human breast malignancies for invasive and metastatic phenotypes.

The generation of an antibody against human matrilysin has raised more questions than it has answered. The protein is expressed in the epithelial cells as expected, but appears to be secreted lumenally. This is a strange pattern for an enzyme that degrades extracellular matrix. It does have the capability of being secreted basally as evidenced by the endometrial tissue. What function does lumenal secretion of matrilysin serve? One answer may lay in recent data from another model system studied in our lab. In the mouse prostate matrilysin is induced following castration and the protein appears to be secreted apically (data not shown). Following castration the prostate involutes as the functional glandular epithelium undergoes apoptotic cell death (20). In matrilysin knockout mice this process is slowed and the apoptotic index is decreased approximately 4 fold. MMPs have been implicated in the formation of soluble Fas ligand which can induce cells expressing the Fas receptor to undergo apoptosis (21). We are currently looking at the possibility that MMPs can function in regulating epithelial cell apoptosis as well as regulating the resolution of inflammatory responses. It is possible that in the normal breast certain lobular units and ducts cycle their epithelial cells and express matrilysin to solublize Fas ligand and induce apoptosis. This could be the same mechanism at work in the normal ducts near expanding lesions, the pressure of the lesion could be inducing a suicide response in the surrounding epithelium. Expression of matrilysin in invasive tumors could be due to the tumor cells escaping Fas mediated apoptosis as was recently shown (22) and trying to compensate by constitutively expressing matrilysin.

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